REMARKS

Claims 1-15 and 17-26 are pending. Claims 3-9 and 19-26 are withdrawn from consideration. Claims 1, 2, 10-15, 17, and 18 are under examination.

Applicants respectfully request that the Examiner indicate whether the replacement drawings filed on November 5, 2004, have been accepted.

Applicants also respectfully request that the Examiner initial and return the Form PTO/SB/08, which was submitted with the Information Disclosure Statement (IDS) filed November 5, 2004, to indicate that the listed documents have been considered.

I. Rejection of Claims 1, 2, 10-15, 17, and 18 Under 35 U.S.C. § 112, First Paragraph

The Examiner rejected claims 1, 2, 10-15, 17, and 18 under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled by the specification. Office Action at page 2. Specifically, the Examiner stated that:

> The specification as filed, is enabled for a DNA polymerase from *Pyrococcus furiosis*, but is not enabled for a DNA polymerase from any and all "samples".
>
> The art of biotechnology is a highly unpredictable art and it would be an undue burden for one of ordinary skill in the art to test any and all sources to see if they contained the claimed enzyme. . . .
>
> While applicant's arguments are noted it is not clear which archaeal bacteria produce the claimed enzyme and which do not.

Id. at page 3. Applicants respectfully traverse.

At the outset, the Examiner should withdraw the rejection of claims 17 and 18, because the Examiner agrees that "the specification as filed, is enabled for a DNA polymerase from *Pyrococcus furiosis*." Claims 17 and 18 recite that "the at least one substantially pure archaeal polymerase and/or archaeal polymerase fragment having polymerase activity is *Pfu* DNA polymerase I" (claim 17) or "*Pfu* DNA polymerase II" (claim 18). The term "*Pfu*" is shorthand for *Pyrococcus furiosis*. *See, e.g.*, specification

at pages 16-17, Examples 1 and 2 (describing an exemplary "chromatographic purification of *Pfu* DNA polymerase I" from *Pyrococcus furiosis* cells). Thus, claims 17 and 18 are enabled.

Applicants further submit that the specification enables claims 1, 2, 10-15, 17, and 18 for the reasons set forth in the Amendment and Response filed November 5, 2004, which is incorporated herein by reference, and for the following reasons.

A claim is enabled if one skilled in the art can make and use the claimed invention without undue experimentation. The Examiner is incorrect in asserting that one skilled in the art would need "to test any and all sources to see if they contained the claimed enzyme." To the contrary, one skilled in the art would know which sources contain archaeal polymerase and/or a fragment thereof. For example, one skilled in the art would know that archaebacteria contain archaeal polymerase and/or a fragment thereof as set forth in claim 1. One skilled in the art would also know that a non-archaeal cell, such as a mammalian cell, most likely would not contain archaeal polymerase and/or a fragment thereof, unless that cell was engineered to express recombinant archaeal polymerase and/or a fragment thereof. Armed with that knowledge, one skilled in the art would not need to engage in trial-and-error testing of "all sources," as the Examiner appears to suggest. One skilled in the art would be able to routinely identify sources that contain archaeal polymerase and/or a fragment thereof. Thus, the Examiner has failed to establish that undue experimentation would be needed for one skilled in the art to determine what sources contain archaeal polymerase and/or a fragment thereof.

The Examiner also appears to argue that the specification allegedly does not enable obtaining an archaeal polymerase and/or a fragment thereof from a source other than *Pyrococcus furiosis*, based on the following statements:

> Applicant has only shown in their examples one source of the claimed enzyme, namely, *Pyrococcus furiosis*. . . .
>
> Applicant argues that the specification recites that recombinantly produced archaeal polymerases that are purified by the novel methods of the invention are known but the invention only shows that this type of enzyme was successfully used in the purification system. . . .
>
> Further, the claims are not even limited to the genuses of archaeal bacteria in the instant specification.

Office Action at page 3. By those statements, the Examiner appears to require that the specification show an example of the purification of an archaeal polymerase from a source other than *Pyrococcus furiosis*, e.g., from a non-archaeal cell expressing recombinant archaeal polymerase or from archaebacteria other than *Pyrococcus furiosis*.

Such an example is not required to meet the standard of enablement. The MPEP states that, "the specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation." MPEP § 2164.02 at 2100-187. Whether experimentation is undue is based, in part, on the guidance provided in the specification, the level of skill in the art, and the level of predictability in the art. MPEP § 2164.01 at 2100-185 (8th ed. rev. 2, May 2004). Applicants assert that the specification, coupled with information known in the art, enables obtaining an archaeal polymerase and/or a fragment thereof from a source other than *Pyrococcus furiosis* without undue experimentation.

For example, the specification provides detailed guidance on obtaining archaeal polymerase from *Pyrococcus furiosis*. *See, e.g.*, specification at pages 16-22, Examples 1-3. The specification also describes other genuses of archaea to which the claimed methods may be applied. *See, e.g.*, specification at page 10, lines 9-11. Furthermore, archaeal DNA polymerases share sequence similarity as well as various functional properties with one another. *See, e.g.*, Lasken et al. (1996) J. Biol. Chem. 271:17692-17696 ("Lasken"), at page 17692, col. 1, first full paragraph after the abstract (submitted with the IDS filed January 5, 2001, and enclosed herewith for the Examiner's convenience). Because archaeal DNA polymerases share such features, they are likely to behave similarly when subjected to chromatographic fractionation. Thus, one skilled in the art would predict that a method of obtaining *Pfu* DNA polymerase and/or a fragment thereof (as shown, e.g., in Examples 1-3) could be successfully used to obtain *any* archaeal DNA polymerase and/or a fragment thereof.

Furthermore, if one skilled in the art wished to obtain archaeal polymerase from a source other than *Pyrococcus furiosis*, then one might adapt the specific details of the procedures used in Examples 1-3 for that particular source. However, such adaptations could be determined without undue experimentation by one skilled in the art having knowledge of routine protein purification methods. Such methods are described, for example, in Ausubel, F.M., et al., Current Protocols in Molecular Biology at pages 10.0.10-10.0.14, Figures 10.0.01–10.0.04 (Supplement 44, 1998), enclosed with the Amendment and Response filed October 16, 2003. Indeed, Ausubel discusses methods of protein purification from a variety of sources expressing either natural or

recombinant proteins. *Id.* Thus, the specification, in view of the level of skill and predictability in the art, would have enabled the full scope of the claims.

The Examiner has failed to establish that undue experimentation would be required to practice the method of claim 1. Thus, claim 1 meets the enablement requirement of 35 U.S.C. § 112, first paragraph. Claims 2, 10-15, 17, and 18 ultimately depend from claim 1. Thus, those claims also meet the enablement requirement of 35 U.S.C. § 112, first paragraph. Withdrawal of the rejection of claims 1, 2, 10-15, 17, and 18 under 35 U.S.C. § 112, first paragraph, is respectfully requested.

II. Rejection of Claims 1, 2, 10-15, 17, and 18 Under 35 U.S.C. § 103(a)

The Examiner rejected claims 1, 2, 10-15, 17, and 18 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Bezuglyi et al. (1992) Mikrobiologicheski Zhurnal 54:51-57 (abstract) ("Bezuglyi"), Bernard et al. (1991) J. Chromatography 539:511-516 ("Bernard"), or Grandgenett (1980) J. Virol. 33:264-71 ("Grandgenett"), taken with Arezi et al. (US 2003/0228616 A1) ("Arezi"), Hogrefe et al. (US 2003/0049614 A1) ("Hogrefe"), Bult et al. (US 6,503,729 B1) ("Bult"), or Hjorleifsdottir et al. (US 6,492,161 B1) ("Hjorleifsdottir"). Office Action at page 4.

Specifically, the Examiner alleged that:

> Bezuglyi et al., Bernard et al., or Grandogenett et al. each teach a DNA polymerase being purified using Poly U Sepharose chromatography.
>
> They do not teach specifically to do this with an archaeal polymerase but as shown by Arezi et al., Hogrefe et al., Bult et al., or Hjorleifsdottir et al. these enzymes (archaeal polymerases) are known in the art and are known to be purified.

Office Action at page 4.

Applicants respectfully traverse the rejection for the reasons set forth in the Amendment and Response filed April 12, 2004, and November 5, 2004, which are incorporated herein by reference, and for the following reasons.

To establish a *prima facie* case of obviousness, the examiner must show that there would have been a reasonable expectation of success. *See* MPEP § 2142 at 2100-128. A reasonable expectation of success requires "at least some degree of predictability." *Id.* § 2143.02 at 2100-133. "Evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness." *Id.*

Applicants assert that the Examiner has not shown that one of ordinary skill in the art would have had a reasonable expectation of success with respect to claim 1. Claim 1 recites a method for obtaining archaeal polymerase and/or a fragment thereof "using Poly U Sepharose chromatography." According to Lasken, archaeal DNA polymerases bind tightly to uracil-containing *DNA* ("dUrd-containing DNA"), which inhibits the polymerases. In contrast, uracil-containing *RNA* does not inhibit archaeal polymerases, suggesting that such polymerases do *not* bind tightly to uracil-containing *RNA*. (See Lasken at page 17693, paragraph bridging cols. 1 and 2.) Poly U Sepharose comprises uracil-containing *RNA* (i.e., chains of polyuridylic acid) coupled to the Sepharose matrix. (See specification at page 15, lines 10-11, and The Merck Index 12th ed., Budavari et al. eds. (Whitehouse Station, NJ, 1996) at page 1685 (showing that 5'-uridylic acid comprises ribose, not deoxyribose), enclosed herewith). Thus, there would have been no reasonable expectation of success that archaeal polymerase would sufficiently adhere to Poly U Sepharose, based on the teachings of Lasken. Indeed,

Lasken would have taught away from using uracil-containing RNA, such as polyuridylic acid, to obtain archaeal polymerase and/or a fragment thereof.

Furthermore, Bezuglyi, Bernard, or Grandgenett would not have provided one of ordinary skill in the art with a reasonable expectation of success. Grandgenett reports the alleged purification of a retroviral polymerase fragment having *endonuclease* activity using Poly U Sepharose. *See* Grandgenett, abstract, and page 266, last paragraph, through page 267. Grandgenett does not discuss or suggest using Poly U Sepharose to obtain a substantially pure polymerase fragment having *polymerase* activity. Thus, one skilled in the art would have had no reasonable expectation of success in modifying the teachings of Grandgenett to obtain an archaeal polymerase and/or a fragment thereof having *polymerase* activity.

Bezuglyi and Bernard report the alleged purification of a mycoplasma DNA polymerase and a human DNA polymerase, respectively, using Poly U Sepharose. *See* Bezuglyi and Bernard, abstracts. Bezuglyi and Bernard do not discuss or suggest using Poly U Sepharose to purify an archaeal polymerase and/or a fragment thereof.

Moreover, one skilled in the art would have had no reasonable expectation of success in modifying the teachings of Bezuglyi, Bernard, or Grandgenett to obtain archaeal polymerase and/or a fragment thereof, particularly in light of known differences between archaeal DNA polymerases and non-archaeal DNA polymerases.

For example, the present specification states that "[a]rchaebacterial DNA polymerases possess characteristics often not found in their eubacterial, eukaryotic, and bacteriophage counterparts." (Specification at page 2, lines 17-18.) Lasken also reports that archaeal polymerases, but not non-archaeal polyermerases, bind to uracil-

containing DNA. Lasken remarks that this "unusual property...is apparently unique to the archaea." Lasken at page 17692, col.1, second full paragraph after the abstract. In light of the unusual characteristics of archaeal polymerases, one skilled in the art would not have expected an archaeal polymerase to behave similarly to polymerases from other organisms when subjected to chromatographic fractionation. Accordingly, one skilled in the art would have had no reasonable expectation of success in using Poly U Sepharose to obtain an archaeal polymerase and/or a fragment thereof.

Furthermore, in light of the unusual characteristics of archaeal polymerases, one skilled in the art who wished to obtain an archaeal polymerase and/or a fragment thereof would not have looked to methods for purifying non-archaeal DNA polymerases for guidance. Accordingly, one skilled in the art would not have been motivated to combine Bezuglyi, Bernard, or Grandgenett with Arezi, Hogrefe, Bult, or Hjorleifsdottir, to arrive at the claimed invention.

Accordingly, the Examiner has failed to establish a *prima facie* case of obviousness. Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 2, 10-15, 17, and 18 under 35 U.S.C. § 103(a).

CONCLUSION

Applicants respectfully request the reconsideration and reexamination of this application and the timely allowance of the application. In the event that the Examiner does not find the application allowable, Applicants request that the Examiner contact the undersigned at (650) 849-6778 to set up an interview.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: May 5, 2005

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Archaebacterial DNA Polymerases Tightly Bind Uracil-containing DNA*

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We show that archaebacterial DNA polymerases are strongly inhibited by the presence of small amounts of uracil-containing DNA. Inhibition appears to be competitive, with the DNA polymerase exhibiting ~6500-fold greater affinity for binding the inhibitor than a DNase I-activated DNA substrate. All six archaebacterial DNA polymerases tested were inhibited, while no eubacterial, eukaryotic, or bacteriophage enzymes showed this effect. Only a small inhibition resulted when uracil was present as the deoxynucleoside triphosphate, dUTP. The rate of DNA synthesis was reduced by ~40% when dUTP was used in place of dTTP for archaebacterial DNA polymerases. Furthermore, an incorporated dUMP served as a productive 3′-primer terminus for subsequent elongation. In contrast, the presence of an oligonucleotide containing as little as a single dUrd residue was extremely inhibitory to DNA polymerase activity on other primer-template DNA.

During the last few years, several DNA polymerases have been identified from thermophilic archaebacteria (1–4). The small number of representatives available thus far have shared sequence similarities (5), and the purified enzymes have had several notable properties in common. All are thermostable as anticipated. Each is associated with a 3′→5′ proofreading exonuclease activity, and none have a 5′→3′ exonuclease activity. *Archaea* is a third kingdom distinct from eubacteria and eukaryotes (6, 7) and is thought to be evolutionarily closer to the eukaryotes. Some archaebacterial proteins share strong homology with eukaryotic counterparts such as RNA polymerase; however, some transcription-associated genes are organized in clusters resembling those of eubacteria (8). The archaebacterial DNA polymerases share significant homology with the family B DNA polymerases (5), which include eukaryotic cell DNA polymerases as well as *Escherichia coli* pol[1] II and bacteriophage T4 DNA polymerase.

We report here an unusual property that is apparently unique to the archaea. The presence of uracil in DNA results in a dramatic increase in the binding affinity of the DNA polymerase. We first observed this effect while attempting to carry out polymerase chain reaction protocols that included dUrd-containing oligonucleotides. These oligonucleotides appeared to block all DNA synthesis, suggesting a direct action on the DNA polymerase. Evidence is presented that suggests that the DNA polymerase forms a tight nonproductive complex with dUrd-containing DNA. We speculate on the possibility that this effect is related in some way to the extreme temperatures at which these thermophiles live and that it may serve in a biological function.

MATERIALS AND METHODS

Oligonucleotides—Oligonucleotides were obtained from Life Technologies, Inc. (Table I). All of the oligonucleotides were determined to be substantially free of secondary structure by analysis with the Oligo Primer Analysis Program, Version 5.0 for Windows (National Biosciences, Inc., Plymouth, MN).

Enzymes—Recombinant *Thermus aquaticus* (*Taq*) DNA polymerase, *E. coli* DNA pol I, and T4 DNA polymerase were purchased from Life Technologies, Inc. DNA polymerases from bacteriophage T5 (9), *Desulfurococcus* strain Tok12-S1 (*Dtok*),[2] *Thermotoga neapolitana* (*Tne*),[2] and *Thermus thermophilus* (*Tth*)[3] were cloned and purified at Life Technologies, Inc. Vent (*Thermococcus litoralis*), Vent exo (−), Deep Vent (*Pyrococcus species* GB-D), and 9°N DNA polymerases were from New England Biolabs, Inc., Beverly, MA. *Pyrococcus woesei* (*Pwo*) DNA polymerase was from Boehringer Mannheim. *Pyrococcus furiosus* (*Pfu*) DNA polymerase was from Stratagene, Inc., LaJolla, CA. *E. coli* DNA pol II was a gift from Myron F. Goodman. Human DNA pol α was a gift from Dan Herendeen and Tom Kelly. Sequenase was from U. S. Biochemical Corp. *Thermus flavus* (*Tfl*) DNA polymerase was from Epicentre Technologies Corp., Madison, WI. Ultma DNA polymerase (derived from *Thermotoga maritima*, *Tne*) was from Perkin-Elmer.

Assays for DNA Polymerase Activity and Inhibition—Reactions for Vent, Vent exo (−), 9°N, and *Pfu* DNA polymerases were carried out in 20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin (supplied as a 10 × reaction buffer by New England Biolabs). Reactions for *Pwo* DNA polymerase were carried out in 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, and 2 mM $MgSO_4$ (supplied as a 10 × reaction buffer by Boehringer Mannheim). Sequenase reactions contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 25 mM NaCl. *E. coli* pol II, *Taq*, *Tth*, *Tfl*, *Tne*, Ultma, *Dtok*, and T5 DNA polymerase reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 5 mM $MgCl_2$. *E. coli* Pol I reactions contained 50 mM potassium phosphate (pH 7.5) and 5 mM $MgCl_2$. Polymerase α was in 40 mM Tris-HCl (pH 7.5) and 6 mM $MgCl_2$. T4 DNA polymerase reactions contained 33 mM Tris acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, and 0.1 mg/ml bovine serum albumin. Unless stated otherwise, reactions contained 0.2 mg/ml DNase I-activated salmon testis DNA prepared as follows. Salmon testis DNA (60 mg) (Sigma, type III) and DNase I (200 ng) (Worthington) were incubated in 100 mM Hepes (pH 7.5), 100 mM $MgCl_2$, 100 mM $CaCl_2$, and 5 mg/ml bovine serum albumin in a final volume of 50 ml at 37 °C for 30 min, and the reaction was stopped by incubation for 15 min at 65 °C. This activated DNA was purified by phenol extraction and EtOH precipitation in 3.25 M ammonium acetate. Where indicated, the DNA template for DNA polymerase activity was 1.4 nM (DNA circles) final concentration of primed M13 single-stranded DNA (Life Technologies, Inc.). Reactions contained a 200 μM concentration of each dNTP with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Corp.) at 50 cpm/pmol nucleotide final concentration. Reactions of 50 μl contained 0.5 unit of DNA polymerase and were incubated for 10 min at 70 °C (polymerases from thermophiles) or 37 °C (polymerases from mesophiles). Incorporation of nucleotide into DNA was measured by acid precipitation. To

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[1] The abbreviations used are: pol, polymerase; UDG, uracil DNA glycosylase.

[2] R. Lasken and D. Chatterjee, unpublished result.

[3] A. Rashtchian and R. Lasken, unpublished result.

TABLE I
Oligonucleotides used in this work

dUrd-85-mer
5′-AUAAAAGUCACCUGCAUCAGCAAUAAUUGUAUAUUGUGGAGACCCUGGAACUAUAGGAAUUAAUGAAGGAGAAUUCCGGUCUCCC-3′
dThd-85-mer
5′-ATAAAAGTCACCTGCATCAGCAATAATTGTATATTGTGGAGACCCTGGAACTATAGGAATAATGAAGGAGAATTCCGGTCTCCC-3′
Single dUrd-71-mer
5′-TCCTGCCTTGCTGCTGCCTTCCUTCGCGTCCTTCCTCGCACTGGCCGTCGTTTTACAACGTCGTGACTGGG-3′
dUrd-42-mer
5′-UCAUCGAGCAUGAUCAGGUCGUGACUGGGACGCCAUGUCUGC-3′
Primer-template for kinetic gel assay
5′-CCCAGTCACGACGTTGTAAAACGACGGCCAGTG-3′
3′-GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGCTCCTTCCTGCGCTACCTTCCGTCGTCGTTCCGTCCT-5′

measure inhibition of DNA synthesis, DNA polymerases were preincubated with inhibitors or controls (Table I) for 2 min at the reaction temperature to allow binding of the polymerase to come to equilibrium, and then reactions were started by the addition of dNTPs and activated DNA.

UDG Treatment of Oligonucleotides—145 pmol of the dUrd-85-mer were incubated at 37 °C for 15 min in the presence or absence of 1 unit of UDG (Life Technologies, Inc.) in a 29-μl reaction containing 20 mM Tris-HCl (pH 8.5) and 50 mM KCl. Then 5 pmol of the oligonucleotide were added to 200 μl of Vent exo (−) DNA polymerase reactions containing 100 μg/ml activated salmon testis DNA substrate as described under "Assays for DNA Polymerase Activity and Inhibition." At the time points indicated, 20-μl aliquots of the reaction mixture were added to 5 μl of 500 mM EDTA.

Kinetic Gel Assay for dUTP Incorporation—The assay was carried out essentially as described (10, 11). The primer-template was designed with a single template A 16 nucleotides from the 3′-primer terminus (Table I). The primer and template oligonucleotides were purified by polyacrylamide gel electrophoresis. The primer was end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Corp.) and polynucleotide kinase (Life Technologies, Inc.), annealed to a 2-fold excess of template by heating to 90 °C for 2 min, and then left at room temperature for 1 h. The primer-template was separated from unutilized [γ-^{32}P]ATP with a 1.5-ml Sephadex G-25 column. 20-μl primer extension reactions contained a 90 nM final concentration of primer-template, Vent reaction buffer (see "Assays for DNA Polymerase Activity and Inhibition"); 200 μM concentrations each of dATP, dCTP, and dGTP (Life Technologies, Inc.); and either dUTP (Pharmacia) or dTTP at concentrations ranging from 0 to 20 μM and 0.02 unit of Vent exo(−) DNA polymerase. Reactions were incubated for 5 min at 70 °C in a Perkin-Elmer 9600 thermocycler. Prior experiments demonstrated that these conditions would extend <10% of the primers as required for the analysis of kinetic parameters (12). Reactions were stopped by addition of 100 μl of 32% (v/v) formamide and 17 mM EDTA. This large volume of stop solution gave a 6-fold dilution of the reaction that seemed to improve resolution by electrophoresis; 3 μl were resolved on an 8% polyacrylamide sequencing gel. The gels were analyzed with a Molecular Dynamics PhosphorImager using MultiQuant software version 3.3. Kinetic parameters were calculated as described (11).

Determination of K_I for Uracil-containing DNA—200-μl reactions were carried out as described above for DNA polymerase inhibition except that 2 units of Vent exo (−) DNA polymerase were preincubated for 3 min at 70 °C with 75, 300, 600, or 1500 μM (nucleotide) DNase I-activated salmon testis DNA in the presence or absence of 2.125 μM (nucleotide) dUrd-85-mer, and then the reaction was started by adding dNTPs. Aliquots were removed for assaying at different times. Plots of the reaction time courses were analyzed to determine initial rates, and the data were replotted in a Lineweaver-Burk plot. The K_I was derived from the relationship: slope in the presence of inhibitor = $K_m/V_{max}(1 + [I]/K_I)$ (13).

RESULTS

Inhibition of Archaebacterial DNA Polymerase Activity by a Uracil-containing Oligonucleotide—In the course of studies using dUrd-containing primers as a method for cloning polymerase chain reaction amplified DNA (14, 15), we observed that when archaebacterial DNA polymerases were used, reactions were consistently inhibited. Using an 85-base oligonucleotide containing dUrd (dUrd-85-mer, Table I) we studied the effect of dUrd-containing DNA on archaebacterial DNA polymerases. The dUrd-85-mer inhibited Vent exo (−) DNA polymerase from utilizing a primed M13 single-stranded DNA substrate (Fig. 1). From 2 to 5 pmol of the dUrd-85-mer effectively blocked synthesis by ~2 pmol of DNA polymerase, suggesting exceptionally tight binding of the enzyme to uracil-containing DNA. A control 85-mer with the same sequence but containing dThd instead of dUrd (dThd-85-mer, Table I) did not block the reaction. The dUrd-85-mer gave a similar level of inhibition to Vent exo (+) DNA polymerase, indicating that the 3′→5′ exonuclease activity of the enzyme does not effect the level of inhibition (data not shown). With DNase I-activated salmon testis DNA as the substrate, 16 other DNA polymerases were tested for inhibition by the uracil-containing 85-mer (Table II). All five of the archaebacterial DNA polymerases were inhibited at levels similar to those of Vent DNA polymerase. None of the other DNA polymerases was inhibited including five thermostable eubacterial enzymes derived from *Thermus* and *Thermotoga* spp. Human DNA pol α, *E. coli* pol II, and bacteriophage T4 DNA polymerase were not inhibited in spite of sharing sequence similarities with the archaebacterial enzymes (5). A 42-mer with a sequence unrelated to the dUrd-85-mer and containing 10 dUrd residues (dUrd-42-mer, Table I) was also strongly inhibitory, suggesting that the presence of dUrd in any DNA sequence is sufficient for inhibition. A 71-mer containing a single dUrd substituted for thymine at position 23 (single dUrd-71-mer, Table I) was also strongly inhibitory for Vent exo (−) DNA polymerase. The single dUrd-71-mer inhibited 60% of the activity, while the dUrd-85-mer (which has 22 dUrd residues; see Table I) inhibited 94% of the activity. Globin mRNA had no inhibitory effect. Therefore, uracil in RNA does not have the same effect as dUrd in DNA. Inhibition from the dUrd-85-mer was not reduced when it was annealed with a complementary 85-mer (not containing dUrd) (Fig. 2). In this experiment, the dUrd-85-mer was annealed to an 8-fold excess of the complementary 85-mer before use as an inhibitor to DNA synthesis. The double- and single-stranded forms of the dUrd-85-mer had nearly identical levels of inhibition.

Relief of Inhibition by Treatment with Uracil DNA Glycosylase—As a further control to demonstrate the direct role of uracil in inhibition, the dUrd-85-mer was pretreated with UDG for removal of uracil bases from DNA. Pretreatment with UDG acted to relieve the inhibitory effect of the dUrd-85-mer (Fig. 3). UDG pretreatment of the dThd-85-mer control oligonucleotide (which is not an inhibitor; see Fig. 1) had no effect on the rate of DNA synthesis (data not shown).

Competitive Inhibition of DNA Polymerase by a Uracil-containing Oligonucleotide—To estimate the effect of uracil on Vent exo (−) DNA polymerase, the K_m^{app} was measured for DNase I activated DNA substrate in the presence or absence of a fixed amount of dUrd-85-mer (Fig. 4). The increase in the slope for the Lineweaver-Burk plot in the presence of inhibitor is characteristic of competitive inhibition and indicates that the

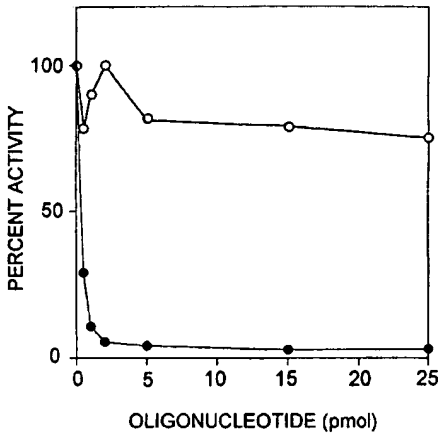

FIG. 1. **Inhibition of DNA synthesis by a uracil-containing oligonucleotide.** Reactions contained Vent exo (−) DNA polymerase and primed M13 single-stranded DNA as a substrate for DNA synthesis as described under "Materials and Methods." The dUrd-85-mer (●) or the dThd-85-mer (○) was added in the amounts indicated.

TABLE II
Comparison of DNA polymerases for inhibition of DNA synthesis by a dUrd-containing oligonucleotide

DNA polymerase	Activity remaining[a]	
	dUrd-85-mer	dThd-85-mer
	%	
Archaea		
1. Vent exo (−)	6	80
2. Deep Vent	14	96
3. *Pfu*	6	87
4. *Dtok*	9	108
5. 9 °N	9	98
6. *Pwo*	9	91
Eubacteria		
7. *Taq*	100	92
8. *Tma*	105	105
9. *Tne*	96	99
10. *E. coli* polymerase I	95	110
11. *E. coli* polymerase II	93	99
12. *Tfl*	95	87
13. *Tth*	98	95
Bacteriophage		
14. T4	95	110
15. T5	118	114
16. Sequenase	94	83
Eukaryotic		
17. Human polymerase α	95	97
	Single dUrd-71-mer	
Vent exo (−)	40	
	dUrd-42-mer	
Vent exo (−)	18	
	RNA[b]	
Vent exo (−)	100	

[a] Determined by duplicate unit assays in the presence and absence of inhibitor as described under "Materials and Methods."

[b] Purified rabbit globin mRNA (Life Technologies, Inc.) was added at a final concentration of 2 μg/ml.

K_I for the dUrd-85-mer is about 6500-fold lower than the K_m^{app} for the activated DNA substrate (see "Materials and Methods").

Utilization of dUTP by Vent DNA Polymerase—A kinetic gel assay (10, 11) was used to determine whether uracil was inhibitory when it was present as the deoxynucleoside triphosphate, dUTP. This experiment allowed the direct measurement of the initial rate of incorporation as distinguished from any

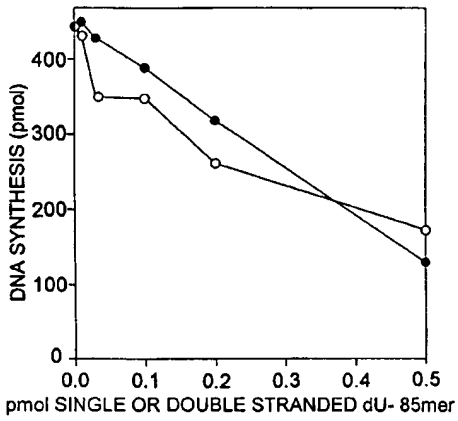

FIG. 2. **Comparison of inhibition by single and double-stranded uracil-containing DNA.** In the presence (●) or absence (○) of an 8-fold excess of the complementary 85-mer (containing no uracil), 5 pmol of dUrd-85-mer were heated to 90 °C for 2 min and cooled for 1 h at room temperature (to allow for annealing) in a 50-μl volume with 1 × Vent reaction buffer and then added in the amount of dUrd-85-mer indicated to Vent exo (−) DNA polymerase reactions as described under "Materials and Methods."

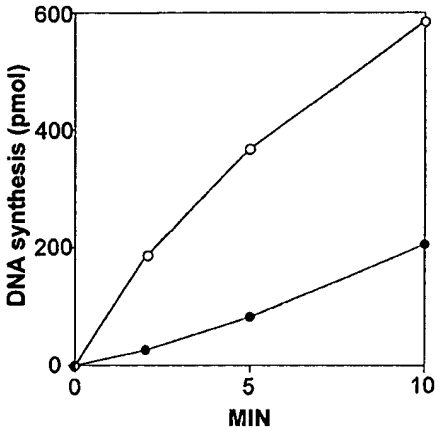

FIG. 3. **Relief of inhibition by UDG treatment of the dUrd-85-mer.** The dUrd-85-mer was incubated for 15 min in the presence (○) or absence (●) of UDG; then its inhibitory effect on Vent exo (−) DNA polymerase using activated DNA as a substrate was determined as described under "Materials and Methods."

subsequent inhibition from the nascent dUrd-containing product which was predicted to be an inhibitor just like the dUrd-85-mer. Vent DNA polymerase was used because Vent is a well characterized representative of archaebacterial DNA polymerases (16), and the exo (−) form was used because enzymes lacking a 3′→5′ exonuclease activity require a simpler analysis (12). A primer-template (Table I) was designed with a single template A target site, designated position N, 16 nucleotides downstream from the 3′-primer terminus. In a reaction containing dATP, dCTP, and dGTP but lacking dTTP, the DNA polymerase rapidly extended the primer by 15 nucleotides but was strongly blocked at the target A. The result is a strong band on a DNA sequencing gel one nucleotide before the target A at position N −1 (Fig. 5*A*, *arrow*). Only a small proportion of the primers were extended past the A due to misincorporated dAMP, dCMP, and dGMP (12). The addition of dTTP or dUTP relieved the block as indicated by the loss of the band at N −1 and the appearance of longer products. The relative rate for dUMP and dTMP incorporation was determined by comparing the concentration of nucleotide required to relieve the block. The relative velocity of incorporation is expressed as the ratio

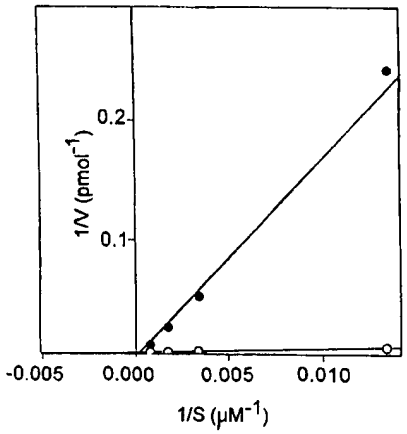

FIG. 4. **Effect of the dUrd-85-mer on Vent exo (−) DNA polymerase's apparent K_m for the DNA substrate.** Initial rates of DNA synthesis were determined for various concentrations of the DNase I-activated salmon testis DNA substrate and then plotted above as described under "Materials and Methods." The dUrd-85-mer was either present (●) or absent (○). In the absence of the dUrd-85-mer, the calculated V_{max} was 0.6 pmol/min, and the apparent K_m was 380 μM (nucleotide).

of the summed band intensities for position ≥N divided by the band intensity at N −1 (10, 11) (Fig. 5*B*). dUMP had an incorporation rate characteristic of a normal nucleotide, being only about 40% less than for dTMP. Therefore, the extreme inhibition from uracil-containing DNA (Fig. 1) is not observed when uracil is present as a deoxynucleoside triphosphate.

The DNA polymerase is also not inhibited immediately after incorporation of dUMP onto the primer terminus, as indicated by the rapid extension of primers past the target A site. If dUMP had been incorporated rapidly but then served as a poor substrate for subsequent extension, this would be revealed by the accumulation of product at the target position N or other downstream positions. No accumulation of these products is observed (Fig. 5*A*). As a further demonstration of this point, a time course of the reaction was carried out with saturating dUTP or dTTP, and the accumulation of full-length runoff products was measured. The full-length products accumulated at the same rate whether dUTP or dTTP was used (Fig. 6). This result requires that dUMP be rapidly incorporated and that subsequent extension of the primer is not blocked. Therefore, uracil at a 3′ terminus does not trap the DNA polymerases in the same way that the uracil-containing 85-mer did (Fig. 1). To be certain that uracil was really the predominant base incorporated at the target A site, some of the runoff products were treated with UDG. More than 90% of these were cut (data not shown).

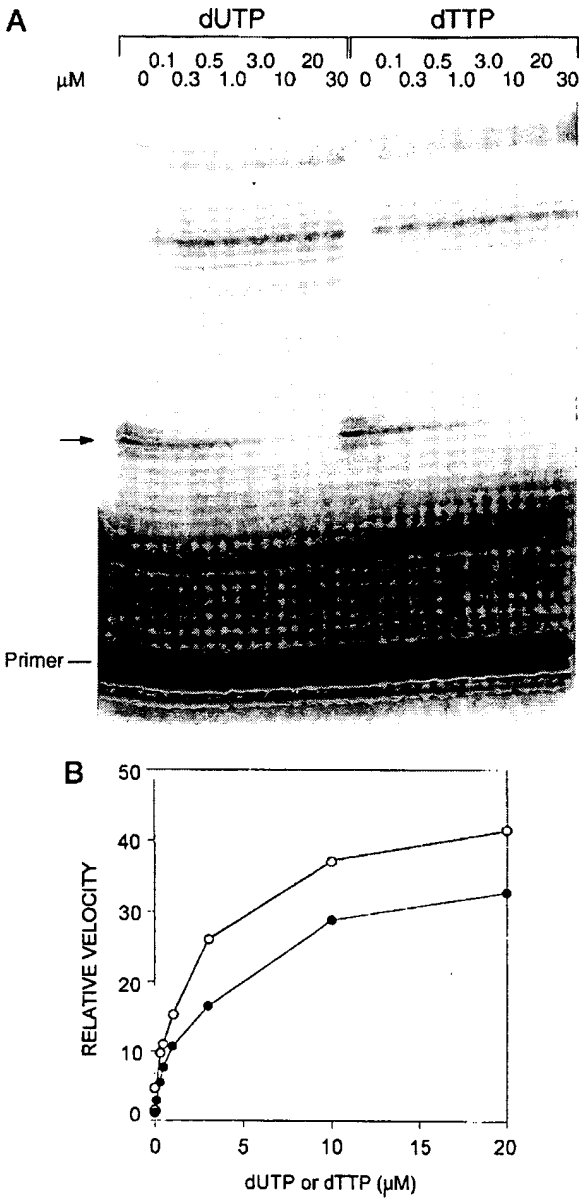

FIG. 5. **Incorporation kinetics of dUTP compared with dTTP.** At the dUTP or dTTP concentrations indicated, 5-min reactions were carried out and resolved on a denaturing polyacrylamide sequencing gel as described under "Materials and Methods." The 5′-^{32}P-labeled primer was annealed to a template with a single A residue 16 nucleotides downstream from the primer terminus (referred to as position N). *A*, at limiting concentrations of dUTP and dTTP, a dark band appears one nucleotide before position N, at position N −1 (*arrow*). The calculated relative velocity of incorporation of dUTP (●) and dTTP (○) are also plotted (*B*).

DISCUSSION

The goal of this study was to identify the nature of the inhibitory effect of uracil-containing DNA and the extent of the effect among DNA polymerases from different organisms. Inhibition of DNA polymerase apparently resulted from an extremely tight, nonproductive binding of the inhibitor. A kinetic analysis indicated a ~6500-fold greater affinity of the DNA polymerase for the inhibitor over the activated DNA substrate (Fig. 4). Levels of activated DNA and the dUrd-85-mer were expressed as concentrations of nucleotides. No attempt was made to control for the difference in the length of the inhibitor (85 nucleotides) and the gapped DNA substrate (average length, several hundred nucleotides). While the measured K_I is specific for this inhibitor, other uracil containing oligonucleotides (Table I) and also an alternative DNA substrate, primed M13 single-stranded DNA, suggested K_I values of similar magnitude. Although every dUrd-containing oligonucleotide tested was inhibitory, the K_I may prove to be influenced by local sequence context as well as oligonucleotide length. The percentage of inhibition for a given concentration of inhibitor decreased with increasing activated DNA substrate consistent with a simple model of competitive inhibition. The extreme preference for binding inhibitor is consistent with the close stoichiometry between inhibitory levels of the dUrd-85-mer and DNA polymerase. Approximately 2 pmol of Vent exo (−) DNA polymerase were almost completely inhibited by 2–5 pmol of the dUrd-85-mer (Fig. 1). The direct role of uracil in the binding was shown by the relief of inhibition by treatment with

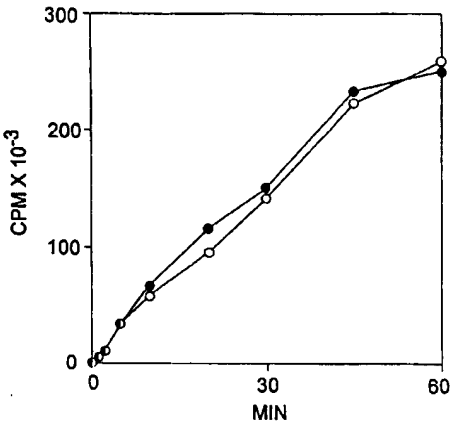

FIG. 6. **Effect of dUTP or dTTP on accumulation of full-length extended primers.** Reactions were the same as in Fig. 5 except that a time course was carried out and the accumulation of full-length runoff products was determined as a measurement of the incorporation and subsequent elongation for saturating dUTP (●) or dTTP (○).

UDG (Fig. 3). Furthermore, an oligonucleotide of the same sequence but containing dThd in place of dUrd was not inhibitory. Even the presence of a single uracil in an oligonucleotide was sufficient for severe inhibition (Table II). Inhibition was about the same for double- and single-stranded dUrd-85-mer DNA (Fig. 2). Globin mRNA had no inhibitory effect. Therefore, uracil in RNA does not have the same effect as dUrd in DNA, although this may simply reflect differences in DNA polymerase affinity between RNA and DNA in general.

We also investigated the effect of uracil in the deoxynucleoside triphosphate, dUTP. A previous study (17) concluded that for Vent and *Pfu* DNA polymerases, dUTP was a poor substrate for PCR polymerase chain reaction and also primer extension in general. Use of the kinetic gel assay (10, 11) allowed us to measure the true polymerization rate for a single dUMP residue at a specific site. This assay is independent of DNA polymerase concentration and should also be independent of any sequestration of polymerase by accumulating uracil containing DNA product. dUTP was utilized fairly normally as a substrate for the Vent exo (−) DNA polymerase, with a rate ~60% of that for dTTP (Fig. 5). Further extension of the primer downstream also occurred at the same rate whether dUrd or dThd was incorporated at the target site (Fig. 6). Therefore, uracil that has just been inserted during ongoing DNA synthesis does not seem to pose the same severe inhibition as seen when an oligonucleotide containing dUrd is presented as an inhibitor to polymerase binding of another DNA substrate. It can also be inferred from these experiments that the presence of dUTP in the reaction does not inhibit incorporation of the normal dNTPs in contrast to the inhibition from the dUrd-containing oligonucleotides. It is likely that the oligonucleotides require some minimal length before the presence of dUrd causes inhibitory binding.

The extremely strong effect of uracil demonstrated here is even more remarkable in that it is seen only for this specific group of DNA polymerases. All archaebacterial DNA polymerases tested were inhibited at similar levels. To date, the number of DNA polymerases available from archaebacteria is still small; and even though three genera of archaea were represented in this study, they may actually represent a fairly small subset of closely related species. The identification of new polymerases will allow a further investigation of how widespread the characteristic is. No eubacterial, eukaryotic, or bacteriophage DNA polymerases were inhibited. Human pol α, *E. coli* pol II, and bacteriophage T4 DNA polymerase were of particular interest because they are reportedly related to the *Archaea* polymerases (5). However, no inhibition was detected. The inhibitory effect is not entirely correlated with the thermostability of the DNA polymerases, since the thermophilic eubacterial enzymes derived from *Thermus* and *Thermotoga* spp. were not inhibited. However, we do note that the archaebacteria involved can generally live at higher temperatures than *Thermus* and *Thermotoga* spp. and may be adapted for more extreme thermostability. Furthermore, it remains possible that the inhibition observed relates to some adaptation involving life at extreme temperatures that is absent in the evolutionarily divergent eubacteria. One possibility is that the strong binding serves a biological role involving the recognition and repair of uracils *in vivo*. In *E. coli*, uracil glycosylase recognizes uracils in DNA and excises them (18). Further research on archaebacteria may reveal whether the binding observed here is involved in this type of repair system. We have searched for primary amino acid sequence homology or conserved motifs in the sequences of known DNA glycosylases and archaebacterial DNA polymerases. There are no obvious conserved motifs in the primary sequence. Although the crystal structure of UDG is known (19, 20), no archaebacterial DNA polymerase structure is available yet.

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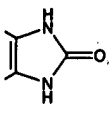

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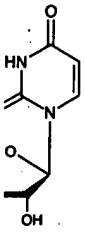

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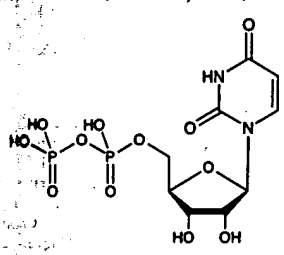

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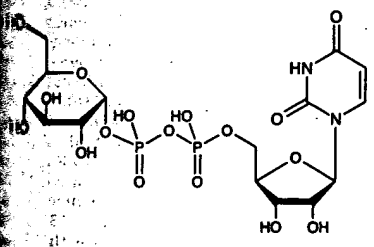
alt trihydrate, $C_9H_{12}N_2O_{12}P_2Na_2.3H_2O$, crystals. pKa' 9.4. uv max (pH 7): 262 nm; (pH 11): reely sol in water.

Uridine Diphosphate Glucose. *Uridine 5'-(trihy- phate) mono-α-D-glucopyranosyl ester;* UDPG; yrophosphate glucose ester; uridine-5'-diphos- o-waldenase; co-galactoisomerase. $C_{15}H_{24}N_2$- wt 566.31. C 31.81%, H 4.27%, N 4.95%, O 0.94%. The coenzyme of the galactowaldenase n catalyzes the conversion of galactose-1-phos- lucose-1-phosphate. Isoln from baker's yeast: l, *J. Biol. Chem.* **184**, 333 (1950). Also present uc. Synthesis: Michelson, Todd, *J. Chem. Soc.* Moffatt, Khorana, *J. Am. Chem. Soc.* **80**, 3756 ews: Leloir, Cardini in *The Enzymes* vol. 2A, P. l, Eds. (Academic Press, New York, 2nd ed., 61; A. M. Michelson, *The Chemistry of Nucleo- cleotides* (Academic Press, New York, 1963) pp W. Hutchison, *Nucleotides and Coenzymes* New York, 1964) pp 36-82.



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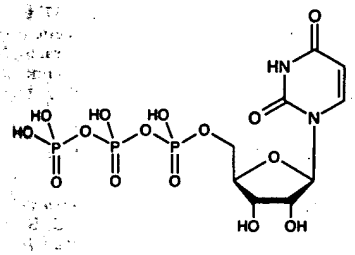
salt dihydrate, $C_{15}H_{23}N_2O_{17}P_2Na.2H_2O$, white uv max (pH 2.0): 262 nm. Freely sol in water.

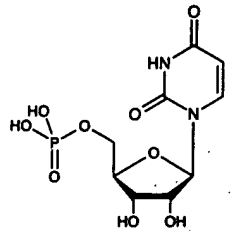
Uridine 5'-Triphosphate. *Uridine 5'-(tetrahydro- phate);* UTP; Uteplex. $C_9H_{15}N_2O_{15}P_3$; mol wt 22.33%, H 3.12%, N 5.79%, O 49.57%, P 19.19%. nalog of ATP. Isoln from rabbit muscle: Lip- *J. Am. Chem. Soc.* **75**, 5450 (1953). Synthesis: l, *J. Chem. Soc.* **1954**, 2288; Hall, Khorana, *J. Soc.* **76**, 5056 (1954). Prepd enzymatically from phosphate or from ribonucleic acid.



Barium salt tetrahydrate, $(C_9H_{12}N_2O_{15}P_3)_2Ba_3.4H_2O$, white powder.

Trisodium salt dihydrate, $C_9H_{12}N_2Na_3O_{15}P_3.2H_2O$, *Utipina.* White powder, sol in water, very sparingly sol in alcohol. pKa_1 6.6; pKa_2 9.5. uv max (pH 7): 262 nm (a_M 10,000); (pH 11): 261 nm (a_M 8100).

10020. 5'-Uridylic Acid. Uridine 5'-phosphoric acid; uridine 5'-monophosphate; UMP. $C_9H_{13}N_2O_9P$; mol wt 324.18. C 33.34%, H 4.04%, N 8.64%, O 44.42%, P 9.55%. Nucleotide; widely distributed in nature. Synthesis by phosphorylation of 2',3'-*O*-benzylidene uridine with diphenyl phosphorochloridate: Brown *et al., J. Chem. Soc.* **1950**, 408; Smith, *Biochem. Prepn.* **8**, 130 (1961). Monograph on the synthesis of nucleotides: G. R. Pettit, *Synthetic Nucleotides* vol. 1 (Van Nostrand Reinhold, New York, 1972) 252 pp. Crystal structure of hydrated barium salt: Shefter, Trueblood, *Acta Cryst.* **18**, 1067 (1965). *Reviews: see* Uridine; Nucleic Acids.



Free acid: pKa_1' 6.4, pKa_2' 9.5. uv max (pH 7.0): 262 nm (a_M 10,000).

Disodium salt dihydrate, $C_9H_{11}N_2Na_2O_9P.2H_2O$, white powder, characteristic meaty taste. uv max (0.1*M* HCl): 262 nm (ε 10,000). Soly in water at 20°: about 41 g/100 ml H_2O.

10021. Urinastatin. *Bikunin trypsin inhibitor;* mingin; urinary trypsin inhibitor; UTI; ulinastatin; Miraclid. Acid-stable glycoprotein (pI 2.8), mol wt ~67,000 by gel filtration. Consists of a single polypeptide chain of 147 amino acid residues containing two Kunitz-type protease inhibitor domains, and two carbohydrate side chains. Normally present in serum and urine. Isoln from human urine: N. R. Shulman, *J. Biol. Chem.* **213**, 655 (1955); and purification: G. J. Proksch, J. I. Routh, *J. Lab. Clin. Med.* **79**, 491 (1972). Characterization: M. Balduyck *et al., Eur. J. Biochem.* **158**, 417 (1986). Protease inhibition: H. Sumi, N. Toki, *Proc. Soc. Exp. Biol. Med.* **167** 530 (1981). Determn by enzyme immunoassay: N. Nishino *et al., Haemostasis* **19**, 112 (1989). Biodistribution and clinical kinetics: B.-M. Jönsson-Berling, K. Ohlsson, *Scand. J. Clin. Lab. Invest.* **51**, 549 (1991). Protective effect in exptl pancreatitis: T. Hirano, T. Manabe, *Arch. Surg.* **128**, 1322 (1993). Clinical evaluation vs ischemic myocardial injury: S. Shimai *et al., Japan. Circ. J.* **53**, 1144 (1989); vs postoperative hyperamylasemia: D. Korenaga *et al., Eur. Surg. Res.* **23**, 214 (1991). Receptor binding studies: H. Kobayashi *et al., J. Biol. Chem.* **269**, 20642 (1994). Review of early literature: H. J. Faarvang, *Scand. J. Clin. Lab. Invest.* **17**, Suppl. 83, 11-77 (1965). Review of characterization as subunit of inter-α-trypsin inhibitor (inter-α-inhibitor): W. Gebhard, K. Hochstrasser in *Proteinase Inhibitors,* A. J. Barrett, G. Salvesen, Eds. (Elsevier, Amsterdam, 1986) pp 389-401; W. Gebhard *et al., Biol. Chem. Hoppe-Seyler* **371**, Suppl., 13-22 (1990).

THERAP CAT: Protease inhibitor.

10022. Urobilins. Bile pigments which occur in feces; and in urine. More specifically described as dipyrrylmethene compds joined at the two α-pyrrolyl positions by methylene bridges to either pyrrolenone or pyrrolidone rings. Reviews of occurrence, proposed structures, stereoisomerism, and interrelationships of *i-urobilin, d-urobilin, stercobilin, urobilin IXα,* and *d-urobilin IXα:* A. H. Jackson *et al., Nature* **209**, 581 (1966); C. H. Gray, D. C. Nicholson, *Medicine* **46**, 83 (1967); W. Rüdiger, *Fortschr. Chem. Org. Naturst.* **29**, 60-139 (1971). Structure of stercobilin and *d*-urobilin: C. H. Gray, D. C. Nicholson, *J. Chem. Soc.* **1958**,